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MiR-214 protects MC3T3-E1 osteoblasts against H_2O_2-induced apoptosis by suppressing oxidative stress and targeting ATF4

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Abstract. – OBJECTIVE: Fragility fracture is one of the common complications of osteoporosis. Elevated oxidative stress-induced apoptosis is thought to be one of the unfavorable factors to osteoblastic dysfunction, which increased the risk of bone fracture. However, the molecular mechanisms for oxidative stress-induced osteoblast cells apoptosis still needs to be elucidated. This study aims to investigate the protective function of miR-214 in H_2O_2-induced apoptosis of MC3T3-E1 osteoblasts.

MATERIALS AND METHODS: MC3T3-E1 cells were treated with 400 μM H_2O_2. Flow cytometry was adopted to detect the apoptosis rate; malondialdehyde (MDA) and glutathione peroxidase (Gpx) levels were used to determine the reactive oxygen species (ROS) level. Reverse transcription-polymerase chain reaction (RT-PCR) was employed to test the expression level of miR-214 and ATF4. After transfected MC3T3-E1 cells with miR-214 mimics and inhibitor, RT-PCR was used to detect activating transcription factor 4 (ATF4) expression level.

RESULTS: H_2O_2 treatment increased ROS induced intracellular oxidative injury. Flow cytometry showed that 400 μM H_2O_2 induced the apoptosis of MC3T3-E1 cells. Moreover, RT-PCR showed decreased expression level of MiR-214. Furthermore, the apoptosis induced by high ROS level was reversed by increased miR-214 expression level. The regulatory ability of MiR-214 to apoptosis is by regulating ATF4 expression.

CONCLUSIONS: miR-214 plays a protective role in H_2O_2 induced MC3T3 osteoblasts apoptosis and its protective effect is proceeded by regulating ROS level and ATF4 expression level.

Key Words:
MiR-214, Apoptosis, ROS, ATF4.

Introduction

Osteoporotic fractures are common in older people, especially postmenopausal women with high morbidity and mortality[1,2]. Osteoporosis is a systemic bone disease caused by a variety of factors leading to decrease of bone mineral density and bone mass and damage of microstructure[3]. Fracture is the main complication of osteoporosis and it is also the most serious stage of osteoporosis. The main reasons of osteoporotic fracture are the decreasing bone strength and increasing bone brittleness. However, osteoporotic fracture cannot be cured. Therefore, the study of pathogenesis and molecular mechanism of osteoporosis has theoretical and clinical significance to prevent the occurrence of osteoporotic fractures and slow down the process of osteoporosis.

The body lives in an environment full of oxygen, and its metabolic process inevitably continues to produce reactive oxygen species (ROS)[4]. ROS would cause damage to the body called oxidation stress when the balance between the ROS generating and eliminating is broken because of aging, disease and other reasons[5]. More and more studies[6] have found that ROS-induced oxidative stress plays an unfavorable role in the pathogenesis of osteoporosis. Excessive ROS regulates several signaling pathways by up-regulation or down-regulation of a variety of cytokines, activation or inhibition of enzyme activities, eventually regulating the gene transcription in cell nucleus, promoting the apoptosis of bone formation associated cells, such as bone marrow mesenchymal stem cells (BMSCs), osteoblasts, bone cells and proliferation and differentiation of osteoblasts[7,8]. So that the bone formation rate relative lagged to bone resorption rate, breaking the dynamic balance between the absorbing bone of osteoclasts and the generating bone tissue of osteoblasts, thereby promoting the production of osteoporosis. At present, the mechanism of oxidative stress promoting osteoblast apoptosis is mainly focused on the regulation of related transcription fac-

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tors. It was found that ROS phosphorylates p66 phosphorylation by activating protein kinase Cβ (PKCβ), then promote osteoblast apoptosis by entering the mitochondria from the cytoplasm and by activating the Jun N-terminal kinase (JNK) signaling pathway[9]. In addition, the study also found that ROS can phosphorylate p66 by the phosphorylation of tumor suppressor p53; then, ROS signal was transformed into the apoptosis of osteoblasts[10]. In this study, we focused on the other mechanism of oxidative stress (OS) caused apoptosis.

miRNAs as one type of important noncoding regulatory factors regulating cell differentiation, proliferation, metabolism, apoptosis and so on biological processes[11-14], could also regulate cellular ROS level[15]. MiR-214 is such a kind miRNA, which has been reported to participate in regulating cell viability and cellular ROS level[16]. Existing result has proved that miR-214 could promote proliferation, and survival in gastric tumors by inhibiting the expression level of phosphatase and tensin homolog deleted on chromosome ten (PTEN)[17]. In addition, miR-214 could inhibit DNA replication and apoptosis by targeting ASF1B, thus increasing the expression of Bax, p53 and p21[18]. The regulation of miR-214 to proliferation and apoptosis has been demonstrated in other studies by directly regulating important genes involved in cell survival[19,20]. It has been shown that miR-214 restrains bone formation and suppress osteoclasts activity by directly targeting activating transcription factor 4 (ATF4)[21]. ATF4 is a vital transcription factor involved in cellular protection, such as oxidative stress and unfolded protein response[22]. In the ROS accumulation induced osteoblasts apoptosis, miR-214 might act as a protective role by regulating its target gene ATF4.

In this report, we focused on the protective effect of miR-214 in H_2O_2 induced osteoblasts apoptosis and involved mechanism. The results showed that the transcription level of miR-214 was decreased in H_2O_2 treated MC3T3-E1 osteoblasts accompanied with the increased ROS level. In order to find the mechanism of ROS caused osteoblasts apoptosis, we focused on the decreasing transcription level of miR-214 in MC3T3-E1 cells. After restoring MiR-214 expression in MC3T3-E1 cells before H_2O_2 treated, the apoptosis rate was reversed. Further studies have shown that the apoptosis protective effect of miR-214 was achieved by regulating ATF4.

Materials and Methods

Chemicals and Materials

H_2O_2 was purchased from Sigma-Aldrich (St. Louis, MO, USA). MC3T3-E1 cells were obtained from ATCC (Manassas, VA, USA). Lipo 3000 Transfection Reagent was obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Gibco (Rockville, MD, USA). Apoptosis Assay Kit was obtained from Keygen (Nanjing, Jiangsu, China). Malondialdehyde (MDA) and glutathione peroxidase (GPx) determination kits were obtained from Nanjing Jiancheng Biochemistry Co. (Nanjing, Jiangsu, China). β-actin and ATF4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

MC3T3-E1 Cell Culture

Murine osteoblastic MC3T3-E1 cells were maintained in a modified minimum essential medium (MEM) media with 10% fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), 2 mM L-glutamine, and 25 μg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO_2. In order for exponential growth, MC3T3-E1 cells were passaged every 2-3 days.

RNA Isolation and Real-time PCR

After MC3T3-E1 cells were stimulated with H_2O_2 as described above, cells were collected by centrifugation, the supernatant was discarded and RNA was extracted with TRIzol reagent. Extraction procedures were carried out according to the operating instructions, RNA was extracted and analyzed by UV spectrophotometer for RNA quantification and purity. The integrity of RNA was detected by 0.8% agarose gel electrophoresis. Afterwards, 1 μg of total RNA was taken by ABI reverse transcription kit according manufacturer's instructions. The reverse transcription reaction program was as follows: 25°C, 10 min; 37°C, 120 min; 85°C, 5 min. Real-time PCR amplification protocol was proceeded according to Roche manufacturer's instructions (Basel, Switzerland).

Western Blot Assay

After MC3T3-E1 cells were treated with H_2O_2 as described above, cells were collected by centrifugation, and the supernatant was discarded. Cell pellets were suspended in cell lysis buffer containing protease inhibitors. The supernatant of cell suspension was separated on sodium do-

decyl sulfate (SDS)-polyacrylamide gels after it was boiled for 5 min in SDS-loading control. Subsequently, the protein was transferred in gel to polyvinylidene fluoride (PVDF) membranes. In order to block nonspecific protein-protein interactions, PVDF membranes were blocked in TST buffer containing 5% non-fat milk at room temperature. After diluting the antibodies (ATF4, 1: 3000; β-Actin, 1: 5000) in TST buffer, the PVDF membranes were gently shaken in the diluted antibodies overnight at 4°C. After washing with TST buffer, PVDF membranes were incubated with peroxidase-conjugated individual secondary antibodies shaking for 1 h. Eventually, the electrochemical luminescence (ECL) solution was prepared in the dark room. The exposure time was determined according to the fluorescence intensity.

Cell Apoptosis Assay

MC3T3-E1 osteoblasts were seeded in a 6-well cell culture dish and Dulbecco Minimum Essential Medium (DMEM) / F12 medium with 10% fetal bovine serum (FBS) was added. After cells adhered to the medium containing different concentrations of H_2O_2, cells were cultured for 24 h and digested with 0.25% trypsin to collect the cells. H_2O_2 treated MC3T3-E1 cells were washed twice with pre-cooling PBS to re-suspend the cells to 10^6/ml. 100 μL of cells were put in a flow tube, then 5 μL of Annexin-V-FITC and 5 μL of pyridine iodide were added. After incubation for 15 min in the dark, 400 μL binding buffer was added to each group, and the fluorescence intensity was measured by flow cytometry in 1 h.

ROS Measurement

MC3T3-E1 osteoblasts were seeded in a 6-well cell culture dish and DMEM / F12 medium was added. After cells were adhered to the medium containing different concentrations of H_2O_2, cells were cultured for 24 h and digested with 0.25% trypsin to collect the cells. H_2O_2 treated MC3T3-E1 cells were washed twice with pre-cooling PBS. Next, cells were broken with chemical buffer and followed protein concentration determination by Bradford method. Following the manufacturer's instructions of the MDA and GPx determination kits (Nanjing Jiancheng Biochemistry Co, Nanjing, Jiangsu, China), the concentration of MDA and the activity of GPx were detected by spectrophotometer.

Statistical Analysis

Each experiment in this article was repeated at least in triplicate. Results were showed as the mean value ± standard deviation (SD). Statistical analysis was carried out using Student's *t*-test. Each *p* value less than 0.05 is thought to be with significance.

Results

H_2O_2 Induced MC3T3-E1 Apoptosis and Elevated Oxidative Stress

H_2O_2 is commonly used in oxidative damage model, in order to select the appropriate concentration of H_2O_2 to MC3T3-E1 cells; several doses of H_2O_2 were tested and 400 μM was the most suitable concentration. After adding 400 μM H_2O_2 to MC3T3-E1 cells for 24 h, cells were harvested for the detection of apoptosis rate. The result showed that H_2O_2 significantly increased the percentage of apoptotic cells compared with solvent control (Figure 1). At the same time, the MDA level, that is an indicator of oxidative damage in H_2O_2 treated osteoblast cells, was significantly increased (Figure 1). Besides, the Gpx level, which reflects the antioxidant level, was decreased in H_2O_2 treated group (Figure 1). This result indicates H_2O_2 mediated oxidative stress impair cell survival in MC3T3-E1 cells.

MiR-214 was Decreased and ATF4 was Increased in H_2O_2 Treated MC3T3-E1 Cells

To identify how oxidative stress-induces apoptosis in MC3T3-E1 cells, we focused on the expression level of miR-214 and ATF4, which all could regulate ROS levels in previous reported articles. We treated MC3T3-E1 cells with 400 μM H_2O_2 for 6 h, and then detected the expression level of miR-214 and ATF4. The expression level of miR-214 detected by Real-time PCR significantly decreased in H_2O_2 treated MC3T3-E1 cells compared with controls (Figure 1). While the expression level of ATF4 significantly increased in H_2O_2 treated osteoblast cells (Figure 1).

MiR-214 Decreased H_2O_2 Induced ROS Level in MC3T3-E1 Cells

The previous report showed miR-214 could regulate ROS levels by different mechanism. To elaborate the mechanism of decreased miR-214 in MC3T3-E1 cells in response to oxidative stress, we transfected MC3T3-E1 cells with miR-214 mimics before treated MC3T3-E1 cells with 400

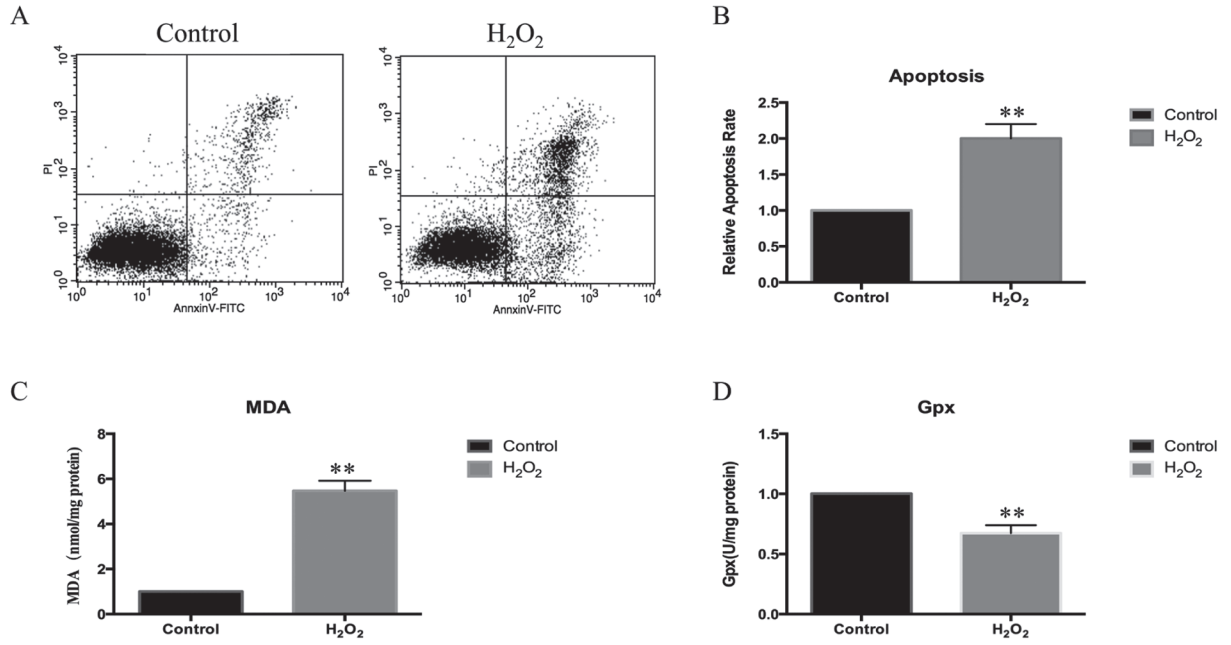

Figure 1. The apoptosis rate and ROS level in H_2O_2 stimulated MC3T3-E1 cells. MC3T3-E1 cells were treated with 400μM H_2O_2 for 24h, the cells were harvested for apoptosis detecting. The results showed H_2O_2 increased the apoptotic percentage ***(A-B)*** elevated MDA level ***(C)*** and decreased Gpx level ***(D)*** in MC3T3-E1 cells.

μM H_2O_2 for 24 h. miR-214 mimics significantly decreased the ROS level and increased GPx level compared with transfected control (Figure 2). The result suggests that miR-214 might play a vital role in regulating MC3T3-E1 cellular ROS level.

MiR-214 Partly Protected Osteoblasts from H_2O_2 Induced Apoptosis

MiR-214 plays a protective role in ROS mediated MC3T3-E1 cell apoptosis. We transfected MC3T3-E1 cells with miR-214 mimics before treatment of MC3T3-E1 cells with 400 μM H_2O_2 for 24 h. The results showed that miR-214 mimics significantly restored the apoptosis rate (Figure 3). The protective effect of miR-214 in H_2O_2 induced MC3T3-E1 cells apoptosis due to its function in reducing cellular ROS level.

ATF4 is Regulated by MiR-214

To investigate the specific mechanism of miR-214 in protecting MC3T3-E1 osteoblasts from

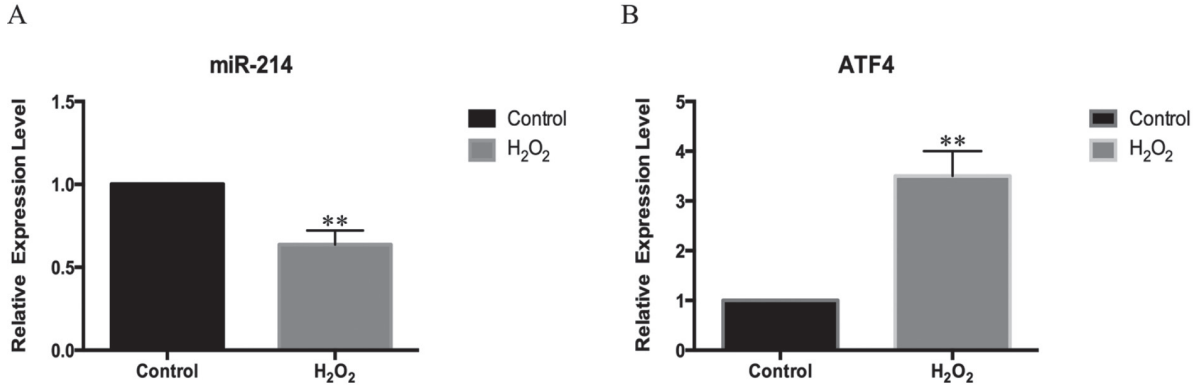

Figure 2. Expression levels of miR-214 and ATF4 in H_2O_2 stimulated MC3T3-E1 cells. RNA was extracted from MC3T3-E1 cells treated with 400 μM H_2O_2 for 6h. The miR-214 and ATF4 expression level were detected by Real-time-PCR. The result showed miR-214 expression level significantly decreased ***(A)*** and ATF4 gene expression level significantly increased ***(B)*** compared with solvent control.

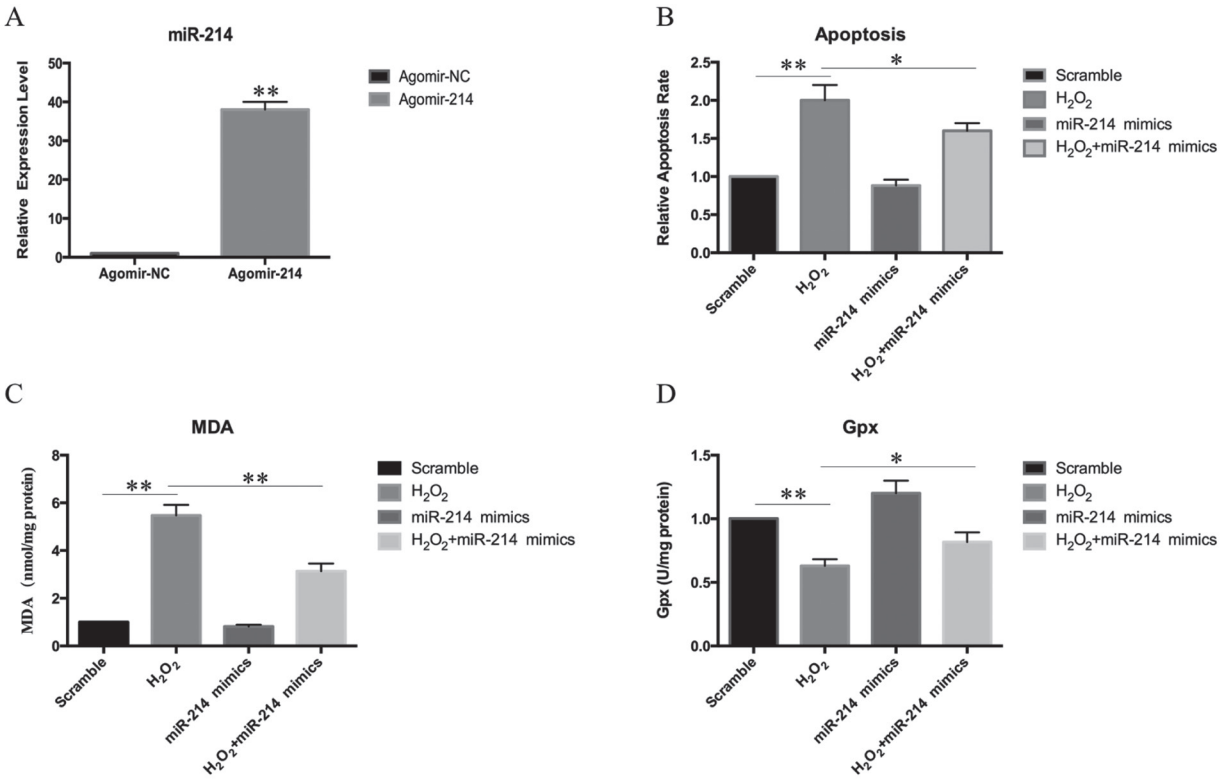

Figure 3. Overexpression of MiR-214 inhibited ROS level and induced apoptosis. MC3T3-E1 cells were transfected with miR-214 mimics, the mRNA level of miR-214 was increased compared with control ***(A)***. MC3T3-E1 cells were transfected with miR-214 mimics before treated with H_2O_2 for 24h, the cell apoptosis rate decreased ***(B)***, the MDA level decreased ***(C)*** and Gpx level increased ***(D)*** in combined group compared with the group of H_2O_2.

H_2O_2 induced apoptosis, we tried to find the downstream target gene of miR-214. According to a recent report, miR-214 could target ATF4 to protect erythroid cells against oxidative stress[17]. In addition, miR-214 was reported in regulating bone formation by targeting ATF4[22]. Besides, the expression level of ATF4 increased in H_2O_2 treated MC3T3-E1 cells in this research. Therefore, in order to identify whether ATF4 could be regulated by miR-214, we transfected miR-214 mimics and inhibitor in MC3T3-E1 cells. After transfecting for 24 h, Q-PCR was applied to detect ATF4 expression level. As shown in Figure 4, ATF4 expression level decreased in miR-214 mimics transfected cells and increased in miR-214 inhibitor transfected cells. The result is consistent with other studies, which showed ATF4 was regulated by miR-214.

Inhibition the Expression of ATF4 Reversed the Apoptosis Rate Caused by H_2O_2

ATF4 is a transcription factor and plays an essential role in regulating cell survival[17,24]. The expression level of ATF4 was significantly increased in H_2O_2 treated MC3T3-E1 cells. Therefore, we hypothesized that ATF4 could promote the process of apoptosis. We constructed ATF4 overexpression plasmid and transfected to MC3T3-E1 cells. Western blot confirmed that ATF4 over-expression plasmid worked efficiently. Transfected the cells with ATF4 overexpression plasmid before treatment MC3T3-E1 cells with H_2O_2. As shown in Figure 5, overexpression of ATF4 completely restored the apoptosis rate of MC3T3-E1 cells treated by H_2O_2 for 24 h. This result suggested miR-214 protected MC3T3-E1 cells from oxidative damage by regulating ATF4.

Discussion

The prevention of the damage caused by high-level reactive oxygen species (ROS) is an important strategy in osteoporosis induced fragility fracture. The study of the mechanism involved in the oxidative stress-induced osteoblast

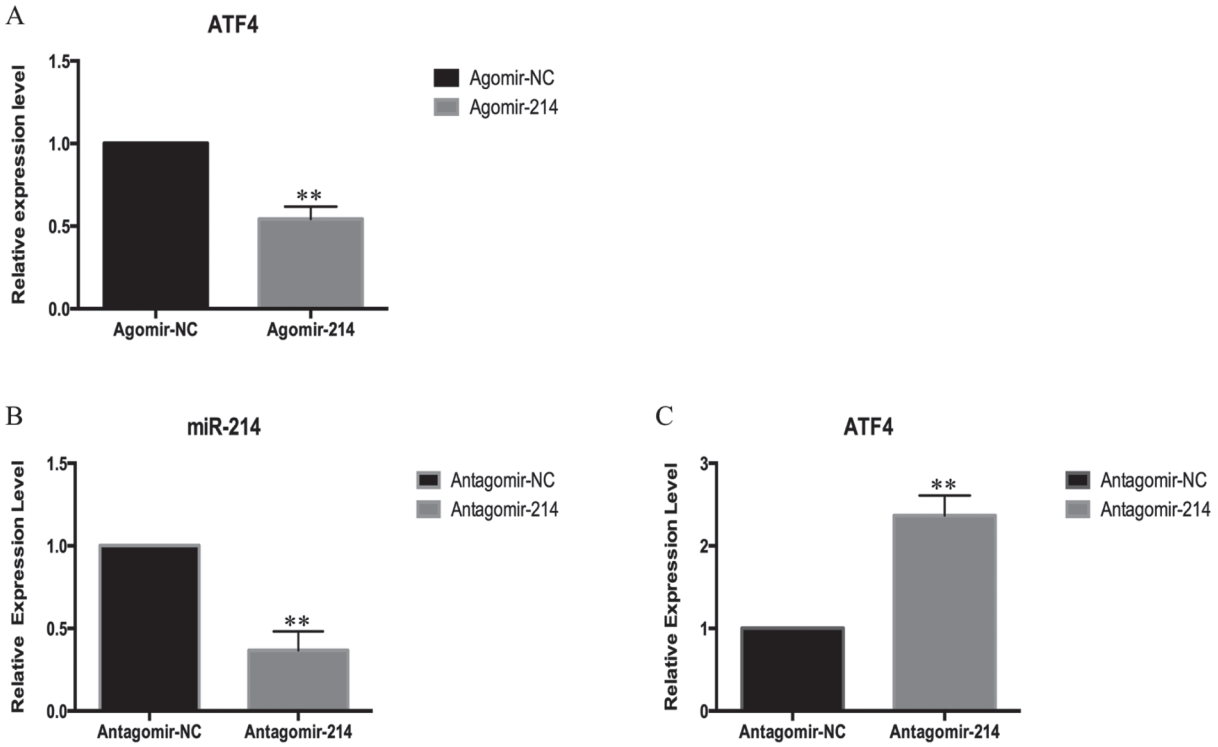

Figure 4. MiR-214 regulated the transcription level of ATF4. MC3T3-E1 cells were transfected with miR-214 mimics and inhibitors, the ATF4 mRNA level was detected by Real-time-PCR. ***A,*** The ATF4 expression level was significantly decreased when MC3T3-E1 cells were transfected with miR-214 mimics. ***B,*** The ATF4 expression level was significantly increased when MC3T3-E1 cells were transfected with miR-214 inhibitors.

cells apoptosis is important to the prevention of osteoporosis induced bone fracture. In this study, miR-214 could protect the oxidative stress-induced osteoblast cells apoptosis in H_2O_2 induced oxidative damage cell model. The protective effect of miR-214 was achieved by decreasing ROS level and targeting ATF4 expression, which is a novel mechanism of osteoporosis, induced fragility fracture.

We demonstrated a novel mechanism of miR-214 in protecting cells from oxidative stress-induced cell apoptosis by targeting the ATF4 gene

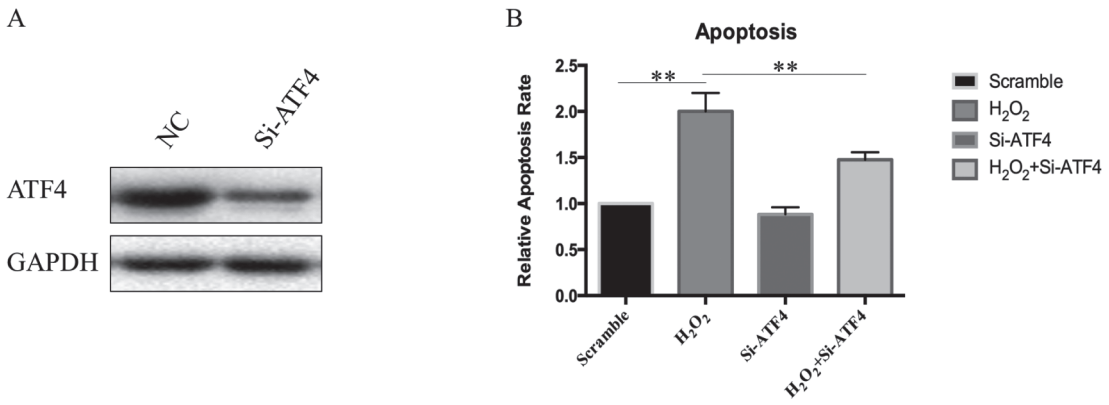

Figure 5. Inhibition the expression of ATF4 reversed the apoptosis rate caused by H_2O_2. MC3T3-E1 cells were transfected with ATF4 siRNA, the protein level of ATF4 was decreased compared with control ***(A)***. MC3T3-E1 cells were transfected with ATF4 siRNA before treated H_2O_2 for 24h, the cell apoptosis result showed the apoptosis rate was significantly decreased in the combined group compared with the H_2O_2 group ***(B)***.

in MC3T3-E1 cells. The protective effect of miR-214 would be used in the treatment of osteoporosis and prevent the caused damage such as fragility fracture. According to previous studies, the expression level of miR-214 was decreased during fracture healing[16]. In this study, miR-214 might play an essential role in the oxidative stress-induced bone cell apoptosis. Oxidative stress as a risk factor for osteoporosis has recently earned more and more attention. Estrogen deficiency and aging are two important pathogenic factors of osteoporosis, both of which can lead to increased levels of oxidative stress in the body[24,25]. A number of studies have found that oxidative stress could promote the development of osteoporosis through the consumption of antioxidants, inhibition of bone marrow osteoblast differentiation and bone formation, promotion of osteoclast differentiation and effect of bone metabolism[8,26]. Therefore, the administration of antioxidants can protect bone cells from osteoporosis and may also be helpful to accelerate fracture healing. Therefore, the antioxidant ability of miR-214 may be used in prevent of osteoporosis and fracture healing.

MiR-214 has many different functions involved in multiple signaling pathways that regulate multiple biological processes of cells. In different models, miR-214 exhibits different or even opposite function. On the one hand, miR-214 might have different target genes in different cells or tissues; on the other hand, the different effect of miR-214 might be caused by the different functions of the downstream target gene in different cells or tissues. For example, although TFAP2 factor is a clearly defined tumor suppressor, it is often lost its expression during melanoma progression[27]. However, its role in breast tumor is different. In fact, TFAP2 was upregulated in ErbB2/HER2-positive breast cancer and induced ErbB2/HER2 expression[28,29]. However, other studies have also observed a decrease in transcriptional levels of TFAP2 in high metastatic breast cancers[30]. In our study, miR-214 inhibited H_2O_2-induced apoptosis of MC3T3-E1 cells by inhibiting the production of ROS.

MiR-214 regulates the ROS level by targeting ATF4, which is one of the mechanisms of H_2O_2-induced apoptosis of MC3T3-E1 Osteoblasts. In the study of ROS induced cardiac myocyte injury, miR-214 could protect cardiac myocytes against injury via targeting PTEN[31]. In ovarian cancer and gastric cancer, PTEN is regulated by miR-214 both at the mRNA level and protein level[32,33]. PTEN is a dual protein phosphatase which inhibits PI3K/Akt signaling pathway to regulate cell survival, growth and so on[34,35]. In fracture healing mice model, the mice lacking PTEN in osteoblast have late endochondral fracture healing[36]. The specific mechanism of PTEN in ROS mediated MC3T3-E1 cell apoptosis still need to be verification. Dysregulation of the other target genes of miR-214 might also participate in the regulation of H_2O_2-induced apoptosis of MC3T3-E1 osteoblasts.

Conclusions

We demonstrated the protective mechanism of miR-214 against H_2O_2-induced apoptosis of MC3T3-E1 osteoblasts. The decreased expression level of miR-214 and increased expression level of ATF4 in H_2O_2 treated MC3T3-E1 cells induce the apoptosis pathway further.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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